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Regulation of *Saccharomyces cerevisiae* CDC7 Function During the Cell Cycle

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The yeast Cdc7 function is required for the G1/S transition and is dependent on passage through START, a point controlled by the Cdc28/cdc2/p34 protein kinase. *CDC7* encodes a protein kinase activity, and we now show that this kinase activity varies in the cell cycle but that protein levels appear to remain constant. We present several lines of evidence that periodic activation of CDC7 kinase is at least in part through phosphorylation. First, the kinase activity of the Cdc7 protein is destroyed by dephosphorylation of the protein in vitro with phosphatase. Second, Cdc7 protein is hypophosphorylated and inactive as a kinase in extracts of cells arrested at START but becomes active and maximally phosphorylated subsequent to passage through START. The phosphorylation pattern of Cdc7 protein is complex. Phosphopeptide mapping reveals four phosphopeptides in Cdc7 prepared from asynchronous yeast cells. Both autophosphorylation and phosphorylation in trans appear to contribute to this pattern. Autophosphorylation is shown to occur by using a thermolabile Cdc7 protein. A protein in yeast extracts can phosphorylate and activate Cdc7 protein made in *Escherichia coli*, and phosphorylation is thermolabile in *cdc28* mutant extracts. Cdc7 protein carrying a serine to alanine change in the consensus recognition site for Cdc28 kinase shows an altered phosphopeptide map, suggesting that this site is important in determining the overall Cdc7 phosphorylation pattern.

INTRODUCTION

The eukaryotic cell cycle is composed of two major events, S phase, during which the chromosomes are replicated, and M phase, during which the chromosomes are segregated. These two portions of the cycle are separated by gaps called G1 and G2, which are devoted to regulating the transitions between replication and segregation. During G1, the events leading to chromosome duplication are monitored and executed. Checkpoints ensuring completion of DNA synthesis and readiness for nuclear division occur in G2 (Hartwell and Weinert, 1989). In the past few years, significant breakthroughs have been made in determining the molecular events that regulate and promote entry into M phase (Dunphy and Newport, 1988a; Lewin, 1990). A key regulatory molecule in this process is highly conserved in eukaryotes, namely, the p34/cdc2$^+$/MPF (mitosis promoting factor) protein kinase subunit (Dunphy and Newport, 1988b). Because much less is known about the events leading to the initiation of nuclear DNA replication at the G1/S transition, we have undertaken studies of this phase of the cell cycle.

In unicellular organisms such as yeast (*Saccharomyces cerevisiae, Schizosaccharomyces pombe*), the major control point in G1 is called START, which may or may not correspond to the "restriction point" in mammalian cells (Pringle and Hartwell, 1981; Pardee, 1989). START defines the point in the cycle after which the cell is committed to a round of DNA synthesis and no longer can undergo the alternative developmental program of conjugation. Several yeast mutants, the best studied of which is *cdc28*, arrest at a point in G1 at which conjugation is still possible and thus are said to affect START (Reed, 1980; Pringle and Hartwell, 1981). *CDC28* encodes the homolog of the p34/cdc2$^+$/MPF protein kinase subunit (Reed and Wittenberg, 1990). Thus it appears that the same protein kinase is involved in regulating both the G1/S and G2/M transitions during the yeast cell cycle (Nurse and Bissett, 1981; Piggott *et al.*, 1982; Ghiara *et al.*, 1991; Surana *et al.*, 1991).

We have very little information on the molecular nature of the events set in motion by *CDC28* and the other START genes. Although the commitment to DNA synthesis is made by passage through START, DNA synthesis does not begin immediately. A number of addi-

tional genetically defined steps must occur before the onset of DNA replication. It is likely that both induction of the genes encoding replication proteins and post-translational activation of replication proteins are preconditions for the initiation of DNA synthesis. The *CDC28, CDC4,* and *CDC7* genes are thought to define one series of steps that must be completed before the initiation of DNA synthesis (Hartwell, 1976; Pringle and Hartwell, 1981). *CDC28* encodes a protein kinase, *CDC4* encodes a protein with homology to the b-subunit of transducin and the ets oncogene (Peterson *et al.*, 1984; Fong *et al.*, 1986) and *CDC7* encodes a protein kinase (Patterson *et al.*, 1986; Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991). Though these activities suggest a signal transduction cascade, little information is available about their functional interrelationships.

Our focus has been to determine the biochemical basis for the position of the Cdc7 protein in the putative G1 regulatory cascade. The *CDC7* controlled events have been shown genetically to depend on completion of START, because the *CDC7* step cannot be completed during block of cells with the mating pheromone α-factor, which like *cdc28* mutations, arrests cells at START (Hereford and Hartwell, 1974). The Cdc7 protein is thought to execute its function late in G1, since cells carrying a thermosensitive (ts) *cdc7* mutation arrest mitotic growth at the G1/S phase boundary at the restrictive temperature, just before the initiation of DNA synthesis (Hartwell, 1973, 1976). These cells show a dumbbell shaped terminal phenotype typically associated with a DNA synthesis or nuclear division defect (Hartwell, 1973). Another reason for placing *CDC7* function very late in G1 is the fact that *cdc28* and *cdc4* mutants blocked at the nonpermissive temperature and then returned to the permissive temperature in the presence of the protein synthesis inhibitor cycloheximide do not progress further through the cycle and do not enter S phase, whereas *cdc7* mutants treated in the same way enter S phase and complete DNA synthesis in the presence of protein synthesis inhibitors (Hereford and Hartwell, 1974). Further protein synthesis is thus necessary for the initiation of DNA synthesis after *CDC28* and *CDC4,* whereas all of the proteins essential for DNA replication appear to be present at the *CDC7* stage. The crucial proteins required have not yet been identified, but the cyclins are likely to be among them (Wittenberg *et al.*, 1990). *CDC7* thus mimics the final stage of the "restriction point" in mammalian cells, insofar as it was defined as the point in the cell cycle after which no further protein synthesis is necessary for initiation of DNA synthesis. Thus *CDC7* may define a second, post-START commitment point in the yeast cell cycle. Evidence suggesting the presence of Cdc7 protein in a putative DNA replication complex has been presented (Jazwinski, 1988).

In contrast to this behavior during mitosis, diploid homozygous *cdc7*-ts cells (like *cdc28*-ts) initiate premeiotic DNA synthesis normally at the restrictive temperature. However, these diploids do not form a synaptonemal complex, nor do they commit to recombination or form ascospores, indicating that the *cdc7* lesion affects the mitotic and meiotic pathways in a different, distinct manner (Schild and Byers, 1978). Furthermore, the *CDC7* gene has also been implicated in an error-prone DNA repair pathway as a member of the *RAD6* epistasis group (Njagi and Kilbey, 1982). Finally, Cdc7 may also be involved in transcriptional silencing of the mating type cassette right (HMR) (Axelrod and Rine, 1991).

At present, little is known about how Cdc7 affects these different DNA processes. A clue as to the molecular function of Cdc7 was afforded by the demonstration that the open reading frame encoding *CDC7* contains the 11 catalytic domains characteristic of protein kinases (Patterson *et al.*, 1986). We and others have shown that the Cdc7 protein isolated from vegetatively growing yeast cells is active as a kinase that phosphorylates histone H1 in vitro (Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991). Two groups have shown by site-directed mutagenesis that Lys 76, which corresponds to the conserved lysine residue found in the ATP binding domain of all protein kinases, is essential for in vivo function, arguing that the kinase activity of Cdc7 is essential for its function in mitosis and meiosis (Buck *et al.*, 1991; Hollingsworth *et al.*, 1992). In addition, the Cdc7 protein is modified by phosphorylation and is located in the nucleus (Yoon and Campbell, 1991). The demonstration that Cdc7 is a protein kinase associated with the nuclei of mitotic cells suggests that Cdc7 may function in the mitotic cell cycle by phosphorylating proteins involved in the initiation of DNA synthesis and/or in the G1/S transition. Recently, Din *et al.* (1990) have shown that both *CDC28* and *CDC7* are required in vivo for phosphorylation of the 34 kDa subunit of the replication protein RP-A, and that this phosphorylation occurs at the G1/S transition. RP-A was originally isolated from human cells in the process of fractionating cellular extracts into components capable of reconstituting SV40 DNA replication in vitro (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). RP-A is single stranded DNA binding (SSB) protein, essential for both the initiation and elongation reactions (Wold and Kelly, 1988; Kenny *et al.*, 1989, 1990). Because of its documented cell cycle specific phosphorylation pattern, RP-A is a good candidate for direct phosphorylation by either the Cdc28 or Cdc7 kinase, although there may be others as well.

Although we had some clues to the role of *CDC7* from previous work, very few clues were available as to what limits *CDC7* function to a specific point in the cell cycle. *CDC7* is not periodically transcribed, and cells carrying a deletion of *CDC7* on the chromosome can

divide up to eight times after loss of a plasmid carrying the *CDC7* gene (Sclafani *et al.*, 1988). Thus periodic activation of Cdc7 kinase rather than abundance may govern its stage-specific function. Other protein kinases are known to be regulated by association with regulatory subunits and/or phosphorylation. In this work we present evidence that Cdc7 function is, at least in part, regulated by phosphorylation.

MATERIALS AND METHODS

Strains, Media, and Plasmids

The genotypes and sources of the yeast strains used were SEY6210, a *his3, leu2, lys2, suc2, trp1,* and *ura3* (S.D. Emr, California Institute of Technology); YC7379, a *ade1, his7, lys2, tyr1, ura3,* and *cdc7-1* (Yoon and Campbell, 1991); CU4-1, a *ade1, ura3,* and *cdc4-1* (A.Y. Jong, University of Southern California); USC6-5, a *leu2, ura3,* and *cdc6-1* (A.Y. Jong, University of Southern California); YC488m, a *his1, leu2, pol1-17, trp1, ura3,* and *can1* (M. Budd, California Institute of Technology); YC28, a *ade1, his3, ura3,* and *cdc28-13* (this work); G1906c, a *bar1-1, leu1, rme1, trp5, ura3, can1,* and *cir*$^+$ (A.Y. Jong, University of Southern California). Cells were routinely grown in yeast extract, peptone, dextrose (YPD) medium (1% yeast extract, 2% Bacto peptone, 2% glucose) or synthetic minimal medium supplemented with amino acids and adenine but lacking uracil. Either 2% glucose, or 2% raffinose, plus 2% galactose were used as the carbon source. For in vivo $^{32}P_i$ labeling, cells were grown in LPSM medium (Reneke *et al.*, 1988). *Escherichia coli* cells were grown in 1% bacto tryptone, 0.5% bacto yeast extract, and 1% NaCl (LB) medium containing ampicillin (50 μg/ml). To overexpress wild-type *CDC7*, thermolabile *cdc7-1*, and the *CDC7*-hemagglutinin fusion gene in yeast, we used a high copy number, 2 μm containing vector pSEY18-Gal (S.D. Emr). pSEY18-Gal also contains the *URA3* gene and the inducible *GAL1,10* promoter. pSYC758 carries wild-type *CDC7* under the *GAL10* promoter of pSEY18-Gal (Yoon and Campbell, 1991). pSYC7-158, carries a temperature sensitive *cdc7-1* gene under the *GAL1,10* promoter, and was constructed for this work as explained in detail below. pSYCHA7 carries the *CDC7*-hemagglutinin epitope fusion gene under the control of the *GAL1,10* promoter (see below for details). The T7 promoter containing vector, pT7-7 (Tabor and Richardson, 1985), was used to express the Cdc7-hemagglutinin epitope fusion protein in *E. coli.* Plasmid pSYC7-83 carries a *cdc7* mutant allele with a Ser 83 to Ala change cloned in pSEY18-Gal exactly as in pSYC758.

Buffers and Inhibitors

The buffers and inhibitors used were the following: C buffer: 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 150 mM NaCl, 15% sucrose; IP buffer (immunoprecipitation buffer), 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.2 mM EDTA, and protease inhibitors; K buffer (kinase buffer), 25 mM Tris-HCl at pH 7.5, 5 mM NaF, 15 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and protease inhibitors; L buffer (lysis buffer), 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.9, 0.5% sodium deoxycholate, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.2 mM EDTA, 0.2 mM EGTA, 2.5 mM dithiothreitol, protease inhibitors, and phosphatase inhibitors; AP buffer (acid phosphatase buffer), 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) at pH 6.0 and protease inhibitors; CIP buffer (calf intestinal alkaline phosphatase buffer), 50 mM Tris-HCl at pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, and protease inhibitors; protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor; phosphatase inhibitors, 10 mM sodium azide, 10 mM sodium fluoride, 10 mM sodium molybdate, 10 mM sodium pyrophosphate, 10 mM potassium phosphate, 1 mM EDTA, and 1 mM EGTA. Okadaic acid was used at 5 μM.

Antibodies

The production of polyclonal Cdc7 antibody is described in Yoon and Campbell (1991). To purify the Cdc7 antibody, an affinity column was made by linking 5 mg of bacterially produced Cdc7 protein to 1 g of CNBr-activated Sepharose as recommended by Pharmacia (Piscataway, NJ). By using this Cdc7 affinity column, the antibody was purified according to the method detailed in Harlow and Lane (1988). The monoclonal antibody 12-CA5, a subclone of H26D08 (mouse IgG 2b), was raised against the influenza hemagglutinin peptide (HA; YPYDVPDYA) as previously described by Field *et al.* (1988). The hybridoma was kindly provided by D.J. Anderson (California Institute of Technology) and was cultured in the Caltech Monoclonal Antibody Facility. The anti-HA peptide antibody was purified from ascites fluid by using a protein A-Sepharose column according to manufacturer's instructions (Sigma, St. Louis, MO). Antibodies to the 11 N-terminal amino acids of Cdc28 as well as the *N*-terminal peptide immunogen were the gift of M. Tyers and A.B. Futcher, Cold Spring Harbor, NY. p13 beads were the gift of W.G. Dunphy, California Institute of Technology, Pasadena, CA.

Immunoprecipitations and Protein Kinase Assays

Either whole-cell or nuclear extracts were used to prepare immune complexes. The amounts of extracts and antisera used are indicated specifically in each Figure legend. Cdc7 immune complexes were prepared as previously described (Yoon and Campbell, 1991). Cdc28 immune complexes were precipitated with 20 μl of protein A-Sepharose beads (100 mg/ml; Sigma), washed four times in IP buffer, twice in K buffer, and resuspended in 25 μl of K buffer. After 5 min preincubation at 25 or 38°C, 0.1 mM ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham [Arlington Heights, IL] PB10168) and substrate were added to a final vol of 36 μl. Incubation was continued at the same temperature for 20 min and reaction products were analyzed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Purification of HA-Cdc7 Fusion Protein From E. coli

Because we had previously shown that the N-terminal 20 amino acids were dispensable for *CDC7* function in vivo (Yoon and Campbell, 1991), we chose to add the hemagglutinin epitope (HA) to the *N*-terminus of the Cdc7 protein. Plasmid pTHA-*CDC7* encoding the hemagglutinin epitope-Cdc7 fusion protein, called HA-Cdc7 in the text, was constructed from the plasmid pT7-*CDC7*. pT7-*CDC7* carries the complete *CDC7* gene cloned into the T7 promoter based vector pT7-7 and has a unique *Nde* I site which marks the initiation codon for *CDC7*. To construct pTHA-*CDC7*, the synthetic oligonucleotide 5'-T ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CC-3' encoding the HA epitope, YPYDVPDYA, and the linker sequence, SLGGP, was inserted into the *Nde* I site of pT7-*CDC7*. The linker GGP is used to connect the HA peptide to the *CDC7* gene because it is expected to cause the HA peptide to protrude from the rest of the protein. Then, the junction sites of recombinant clones were sequenced to confirm the cloning procedures yielded an in-frame fusion gene.

For expression of the HA-Cdc7 fusion protein in *E. coli*, pTHA-*CDC7* was transformed into the bacterial strain BL21 (DE3) (Studier and Moffat, 1986). This strain carries the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Cells carrying pTHA-*CDC7* were grown in 500 ml of LB medium supplemented with ampicillin (50 μg/ml) to an optical density (OD_{600}) of 0.5. The polymerase, and hence the HA-*CDC7* fusion gene, were induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM, and the culture was allowed to grow for another 3 h. Cells were harvested, resuspended in 20 ml of ice-cold 10 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10 μg/ml lysozyme, protease inhibitors, and disrupted by sonication with four 30-s pulses. The inclusion bodies containing the insoluble HA-Cdc7 fusion protein were isolated by centrifuging the bacterial extracts at 10 000 rpm for 10 min at 4°C in a Sorvall (Newton, CT) SS34 rotor. The pellet was

resuspended in 200 mM Tris-HCl at pH 8.2, 500 mM NaCl, 0.1% Triton X-100 (Triton Diagnostics, Alameda, CA), 1 mM dithiothreitol, sonicated once for 30 s, and centrifuged again at 10 000 rpm for 10 min. The final pellet was dissolved in 50 ml of buffer A (25 mM Tris-HCl at pH 8.0, 1 mM dithiothreitol, and 6 M urea).

To purify HA-Cdc7 fusion protein, protein extracts were loaded onto 20 ml of DEAE-Cellulose (Whatman, Clifton, NJ) column that had been equilibrated with buffer A containing 25 mM NaCl. After washing the column with two column volume of buffer A with 50 mM NaCl, proteins were eluted by a linear gradient of 50–500 mM NaCl in buffer A. The HA-Cdc7 fusion protein was eluted at ~250 mM NaCl, as determined by protein blotting of the fractions with Cdc7 antibody. The fractions containing the fusion protein were pooled, diluted to a protein concentration of ~50 ng/μl with buffer B (200 mM Tris-HCl at pH 8.0, 500 mM NaCl, and 1 mM dithiothreitol) containing 6 M urea, and dialyzed against buffer B with 4 M urea for more than 8 h. The concentration of urea in the dialysis buffer was gradually reduced from 4 M to 2 M, 1 M, and finally 0 M. The length of dialysis in each urea concentration was for more than 8 h each. The fusion protein was judged to be >90% pure by Coomassie blue staining following SDS-polyacrylamide gel electrophoresis.

Expression of HA-CDC7 Fusion Gene in Yeast

To produce HA-Cdc7 fusion protein in yeast, pTHA-*CDC7* was first digested with *Nde* I and *Sph* I. The 1.8-kb DNA containing the HA-*CDC7* fusion gene was then treated with T4 DNA polymerase, *Eco*RI linkers were attached, and the fragment was ligated into the *Eco*RI site of the yeast expression vector, pSEY18-Gal. The resulting plasmid, pSYCHA7, carries HA-*CDC7* gene under the control of the inducible *GAL1,10* promoter and is capable of efficiently suppressing the temperature sensitive phenotype of a *cdc7-1* allele at the nonpermissive temperature, 36°C. Thus the epitope does not seem to interfere with Cdc7 function in vivo when fused to the Cdc7 protein.

Cloning of the cdc7-1 Allele and Overproduction of Thermolabile cdc7-1 Protein in Yeast

The mutation in *cdc7-1* has been shown to be a Gly to Ala change at amino acid position 384, between the *Sac* I and *Sph* I sites (Patterson *et al.*, 1986; Hollingsworth *et al.*, 1992). The polymerase chain reaction was performed in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler by using ~150 ng of yeast genomic DNA isolated from strain *cdc7-1* and 5 μg each of the oligonucleotide primers (5′ primer, 5′-TAT AAT GAG CTC AAC CTG CTG TAC ATA ATG ACG-3′, where the *Sac* I site is underlined; 3′ primer, 5′-CTT AAG CGC ATG CCA CCA ATT ATG CTA AAC CGT-3′ in which the *Sph* I site is underlined). The plasmid containing the temperature sensitive allele, pSYC7-158, was constructed by ligation of the 1,393 bp *Sac* I/*Sph* I PCR fragment of the *cdc7-1* allele into the *Sac* I and *Sph* I sites of plasmid pSYC758, which expresses wild-type Cdc7 protein from the *GAL10* promoter (Yoon and Campbell, 1991). This creates an in-frame fusion of the 3′-1,393 bp of the *cdc7-1* allele to the 5′-282 bp of the *CDC7* gene. Clones were transformed into a heterozygous diploid, *CDC7*/*cdc7*::Tn3, carrying a disrupted *cdc7* allele, which was constructed for this test. After sporulation, the spores containing plasmids and disruptions grew but were temperature sensitive. Thus the clone complements a gene disruption and gives a temperature sensitive phenotype. As expected, the clone does not complement *cdc7-1* strains at 36°C. Protein extracts were prepared from a *cdc7-1* strain containing either pSYC758 (wild-type *CDC7*) or pSYC7-158 (ts *cdc7-1*). Immunoblot analysis of these extracts confirmed that the cdc7-1 protein was overproduced at the permissive temperature at the same levels as wild type (unpublished observation).

Site-Directed Mutagenesis of CDC7

Oligonucleotide-mediated, site directed mutagenesis of *CDC7* was performed by means of the Amersham kit (Amersham, Arlington Heights, IL), used according to the manufacturer's instructions. All mutagenesis was verified by sequencing of the complete open reading frame. The oligonucleotide GTTACCTCGGCACCGCAAAGA was used to mutate Ser 83 to Ala. The gene was cloned into pSEY18-Gal for expression in yeast. The resulting mutant protein was designated Cdc7-Ala 83.

RESULTS

The Activity of Cdc7 Kinase Varies in the Cell Cycle and is Dependent on Passage Through START

Demonstration by Sclafani *et al.* (1988) that *CDC7* is not periodically transcribed and that there appears to be sufficient Cdc7 in the cell to support eight cell cycles, indirectly suggested that fluctuation in abundance of Cdc7 does not account for its periodic activity. To directly investigate the levels of Cdc7 protein and Cdc7 kinase activity at different stages of the cell cycle, the activity and abundance of Cdc7 were determined in extracts of synchronized cells. A standard α-factor block and release protocol was followed. As shown in Figure 1, Cdc7 activity, as measured by ability of Cdc7 immune complexes to phosphorylate histone H1, is low in α-factor arrested cells. Sixty minutes after removal of α-factor, Cdc7 kinase activity increases, at the same time as small buds appear. (The subsequent decrease in the 80′ sample is probably due to poor recovery in the immunoprecipitate, because the level is still two- to three-fold higher than the 40′ point.) Cdc7 activity remains high throughout the rest of the cell cycle. Activity then drops sharply, at a position when cells with large buds are maximal, near the end of the first cell cycle. In the second cell cycle after release from α-factor, activity rises again late in G1. Thus Cdc7 activity varies in the second cell cycle with a timing consistent with its function in late G1. The first cell cycle is also consistent, but the first cell cycle after α-factor release is known to be atypical, in that cells must first recover from α-factor and then enter the cycle, a process that can be affected by physiology of the cells. The second cell cycle is a better indicator of cell cycle timing if good synchrony is achieved.

The second important point demonstrated in Figure 1 is that the variations in activity levels are clearly not determined by variations in the abundance of Cdc7 protein, because the levels of Cdc7 protein, as measured by Western blotting, remain constant in all samples (Figure 1).

To precisely define the timing of Cdc7 kinase appearance, we measured Cdc7 kinase activity in immunoprecipitates of nuclear extracts of cells arrested at various stages in the cell cycle either by use of *cdc* mutants or by the use of cell cycle specific inhibitors. Yeast cells were blocked with the mating pheromone α-factor, hydroxyurea, or nocodazole, which inhibit START, DNA synthesis and mitosis, respectively. In addition, cells were arrested at the *cdc28*, *cdc4*, and *pol1* steps, which represent START, G1, and S phase, respectively.

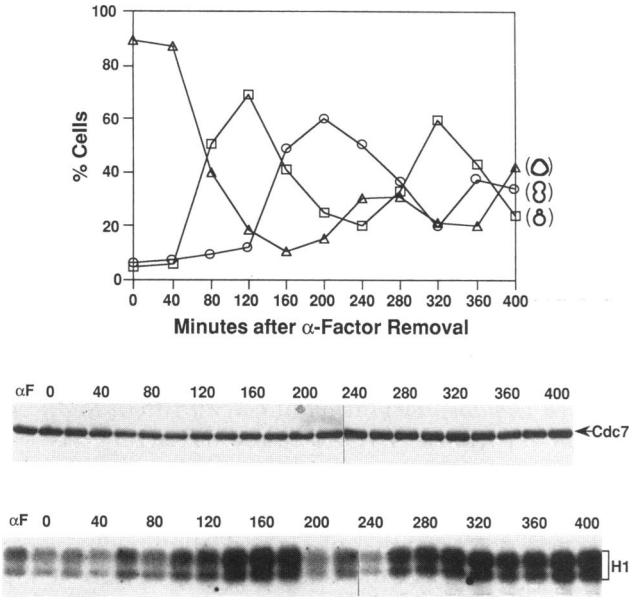

Figure 1. The activity of Cdc7 kinase accumulates periodically during the cell cycle. Strain G1906c *bar1* carrying the *CDC7*-overproducing plasmid, pSYC758, was grown at 30°C in 2 liters of 2% raffinose synthetic complete (SC) medium (minus uracil). At A_{595} = 0.5, cells were treated with 2% galactose and synchronized in G1 with 300 ng/ml α-factor for 7 h. Cells were washed twice with fresh SC medium (minus uracil) containing both 2% raffinose and 2% galactose, and resuspended in 1.5 liters of the same medium to release cells from mating pheromone arrest. Samples (50 ml) were taken at 20-min intervals for 400 min. Cells were harvested and resuspended in C buffer. Whole yeast lysates were prepared from each sample by vortexing in the presence of glass beads and clarified by centrifugation. The supernatants, which contain no detectable Cdc7 protein, were discarded and the pellets were resuspended in L buffer, incubated for 30 min on ice, and centrifuged twice to remove insoluble materials. Protein extracts were analyzed by immunoblotting for Cdc7 quantitation and by histone H1 kinase assay for Cdc7 activity. Protein (40 μg each) blots were stained with Cdc7 antibody (1:250 dilution) and the BioRad ImmunBlot assay kit (goat anti-rabbit IgG alkaline phosphatase conjugate). For histone H1 kinase assay, Cdc7 immune complexes were prepared as described in MATERIALS AND METHODS with 120 μg of protein and 20 μl of Cdc7 antibody. The reaction was carried out at 38°C for 20 min with 5 μg of histone H1, and the products were visualized by autoradiography after separation on 10% SDS-polyacrylamide gels. For parameters of cell cycle synchrony, samples taken at each time point were fixed with 3.7% formaldehyde and scored for cell morphology by phase-contrast microscopy. Symbols: △, unbudded cells; □, cells with small buds; ○, cells with large buds.

As shown in Figure 2, there was no active Cdc7 kinase in *cdc28* blocked cells and greatly reduced, though detectable, levels in α-factor arrested cells (Figure 2, lanes 2 and 6). Protein levels were not significantly reduced (unpublished observation but see Figures 1 and 3B for Western blots of α-factor blocked cells and *cdc28* blocked cells, respectively).

In contrast, cells blocked at the *cdc4* step and beyond in the cycle, even the nocodazole treated cells, contained active Cdc7 kinase (Figure 2, lanes 3, 4, 7, and 8). When *cdc28* cells were grown at the permissive temperature, 25°C, and assayed for kinase activity, normal levels of kinase were observed as shown in Figure 2, lane 5. These findings suggest that the Cdc7 kinase is inactive before START but that passage through START provides a kinase active at least in vitro. Because substrate was provided in the in vitro assay, availability of substrate could not account for the inactivity of Cdc7 in the extracts, although such secondary factors may contribute to the overall regulation of G1 in vivo. Thus we favored the interpretation that activation of the Cdc7 kinase through posttranslational modification during the cycle was an important component of its cell cycle regulation, and sought more direct evidence that this might be the case.

Timing of Activation of the Histone H1 Phosphorylating Activity of Cdc7 Protein Coincides with Increases in in vivo Phosphorylation Levels

We have previously reported that Cdc7 is a phosphoprotein and that this phosphorylation can be detected by in vivo $^{32}P_i$ labeling of cells expressing elevated levels of Cdc7 protein (Yoon and Campbell, 1991). To determine whether the absence of Cdc7 kinase activity coincides with absence of phosphorylation, we carried out blocking experiments similar to those in Figure 2 and characterized the phosphorylation state of Cdc7 during the block. *cdc28*, *cdc4*, *cdc6*, and *pol1* strains were transformed with a plasmid expressing *CDC7* under control of the *GAL1,10* promoter and were labeled with $^{32}P_i$ in vivo during a block at the nonpermissive temperature. [*cdc6* arrests late in G1 with a phenotype similar to *cdc7* (Hartwell, 1976).] As shown in Figure 3, Cdc7 was present as a phosphoprotein in all blocked cells (lanes 2–5). In *cdc28* mutant cells, however, the amount of phosphoprotein was barely detectable in cells held at 37°C as compared with *cdc28* grown at 25°C (Figure 3,

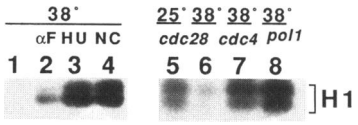

Figure 2. Cdc7 kinase activity in cells arrested at various points in the cell cycle by *cdc* mutations or inhibitors. *cdc* strains (*cdc28-13*, *cdc4-1*, and *pol1-17*) were grown at 25°C and a *bar1* strain at 30°C in YPD media. At OD_{595} = 0.7, *cdc* strains were shifted to 38°C and held there until ~90% of the cells were arrested with the appropriate terminal phenotype (lanes 6–8). *bar1* cells were treated with α-factor (αF; 300 ng/ml final concentration), hydroxyurea (HU; 0.2 M final concentration), or nocodazole (NC; 20 μg/ml final concentration) for 4 h at 30°C (lanes 2–4). Nuclear protein extracts (500 μg) prepared from these strains were immunoprecipitated with affinity purified Cdc7 antibody (20 μl) and assayed for histone H1 kinase activity. The reaction was carried out at 38°C for 20 min with 5 μg of histone H1. As a control, histone H1 was incubated in the absence of Cdc7 immune complexes (lane 1). For the *cdc28-13* strain, nuclear extracts were also prepared from cells grown constantly at 25°C and tested for the histone H1 kinase activity at 25°C for 20 min (lane 5). The reaction products were visualized by autoradiography after separation on SDS-polyacrylamide gels.

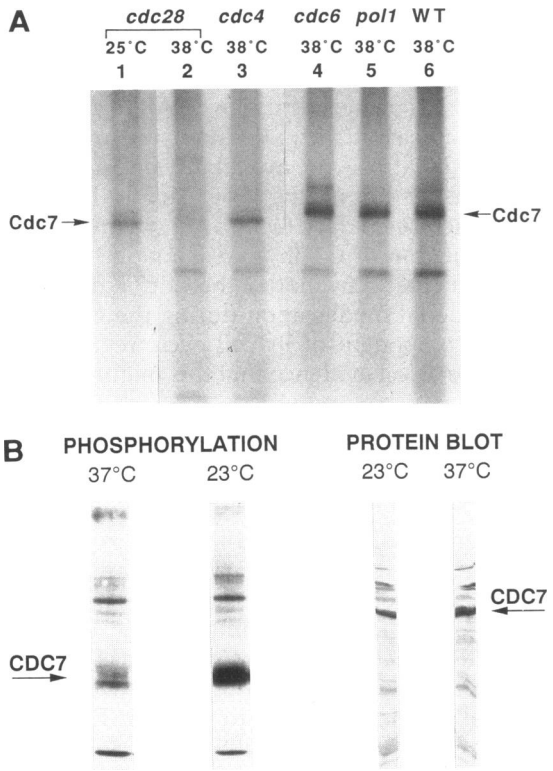

Figure 3. (A) Labeling of Cdc7 protein with $^{32}P_i$ in cells arrested at different points in the cell cycle. Yeast strains (*cdc28-13*, *cdc4-1*, *cdc6-1*, *pol1-17*, and wild type) carrying the *CDC7*-overproducing plasmid, pSYC758, were grown at 25°C in 20 ml of LPSM medium. Both 2% raffinose and 2% galactose were used as a carbon source. At OD_{595} = 0.5, the temperature was shifted to 38°C for 3 h. Cells were then labeled with 0.7–1.0 mCi of $^{32}P_i$ for 2 h at 38°C (lanes 2–6). For the *cdc28-13* strain, cells grown constantly at 25°C were also labeled with $^{32}P_i$ for 2 h at 25°C (lane 1). Whole yeast lysates were prepared as described in the legend to Figure 1. Proteins were immunoprecipitated with affinity purified Cdc7 antibody (20 μl) and analyzed by electrophoresis in a 10% SDS-polyacrylamide gel. The gel was run until the 30-kDa colored marker reached the bottom. (B) Comparison of phosphorylation states and abundance of Cdc7 protein in growing cells and cells blocked by a *cdc28* mutation. Phosphate labeling. $^{32}P_i$ labeling of strain *cdc28* carrying the Cdc7 overproducing plasmid pSYC758 was carried out as described in the legend to Figure 3A, except phosphate depleted YPD with 2% galactose as carbon source (Moll *et al.*, 1991) was used as the growth medium, because the $^{32}P_i$ labeling was found to be more efficient with the Pi depleted YPD. At A_{600} = 0.5, one-half of the culture was shifted to 37°C for 2 h. Cells were then labeled with 0.5 mCi of $^{32}P_i$/ml of growth medium for 2 h at either the restrictive 37° or at 25°C. Protein blot. Approximately 0.5 mg of the cellular extracts used in (A) was blotted onto nitrocellulose for the protein blots and probed with Cdc7 serum which had not been affinity purified.

lanes 1 and 2). Thus there is a positive correlation between when the kinase is active and the phosphorylation state of Cdc7 protein (Figures 2 and 3). These results also show that the kinase that phosphorylates Cdc7 is not limiting and can phosphorylate the Cdc7 protein present in cells expressing elevated levels of Cdc7 protein. This is consistent with the fluctuation in Cdc7 activity during the cell cycle even when Cdc7 is overproduced (Figure 1) and the viability of cells overproducing Cdc7 (Yoon and Campbell, 1991).

To verify that the reduction in level of phosphorylated protein in the *cdc28*-arrested cells was not due to reduction in the amount of Cdc7 protein per se, an independent labeling and immunoprecipitation experiment was carried out in conjunction with a protein blot of the respective extracts. As shown in protein blot in Figure 3B, there is no difference in the abundance of the Cdc7 protein in *cdc28* cells held at 36° or 25°C.

In Vitro Dephosphorylation of Cdc7 Protein Reduces Histone H1 Kinase Activity to pre-START Levels

The positive correlation between the extracts in which Cdc7 protein is maximally phosphorylated and in which it is active predicted that phosphorylation might be essential for activity of the Cdc7 kinase. The histone H1 kinase activity of Cdc7 was therefore monitored after incubating Cdc7 immune complexes in the presence or absence of phosphatase (Figure 4A). For these experiments we used immune complexes prepared from cells overproducing native Cdc7 protein by incubation with Cdc7 antibody (Figure 4A, lanes 1–3). We also prepared a Cdc7-hemagglutinin fusion protein and overproduced it in yeast as described in MATERIALS AND METHODS. Cdc7-containing immune complexes were then prepared with the hemagglutinin monoclonal antibody, 12-CA5, which recognizes the hemagglutinin epitope (Figure 4A, lanes 4–7). The Cdc7 protein immunoprecipitated by the two antibodies was active as a histone H1 kinase (Figure 4A, lanes 1 and 4). Upon phosphatase treatment, however, Cdc7 was inactivated as a kinase, as shown in Figure 4A, lanes 2, 3, and 5. The amount of Cdc7 protein remained constant under the conditions of the phosphatase treatment (Figure 4B), assuring that the inactivation was not due to proteolysis. Although phosphatase was removed by extensive washing before assaying for kinase, two experiments were performed to ensure that there was no residual phosphatase bound tightly to the protein A-Sepharose beads, which might interfere with the Cdc7 kinase. In Figure 4A, lane 6, a combined sample of both phosphatase-treated (1/2) and untreated (1/2) immune complexes is seen to contain about one-half of the kinase activity of that shown in Figure 4A, lane 4, confirming that phosphatase was not dephosphorylating histone H1 during the kinase reaction. In a separate experiment, okadaic acid was added to the kinase reaction following phosphatase removal to inhibit any residual phosphatase. Again, the Cdc7 kinase remained inactive (Figure 4A, lane 7). Thus we conclude that the absence of histone H1 kinase activity in the phosphatase treated Cdc7 protein is due to removal of phosphate(s) important for kinase activity.

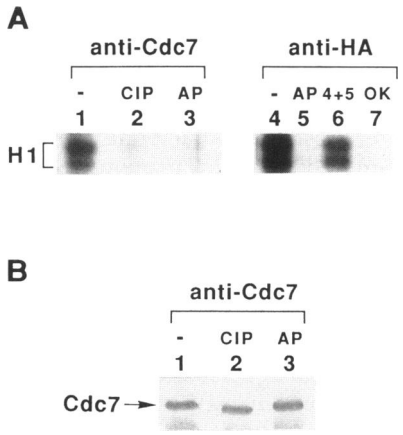

Figure 4. In vitro inactivation of Cdc7 kinase with phosphatase. (A) Three separate Cdc7 immune complexes were prepared from nuclear extracts (500 μg each) of Cdc7-overproducing cells with affinity purified Cdc7 antibody (20 μl each; lanes 1–3). After washing 4 times in IP buffer, 2 of them were washed twice in CIP buffer and incubated for 15 min at 37°C in the presence (+) or absence (−) of 3 U of CIP (lanes 1 and 2). The third immune complex was treated with 3 U of AP after washing twice in AP buffer (lane 3). Each immune complex was then washed 3 times in IP buffer and twice in K buffer, and assayed for histone H1 kinase activity (15 min at 37°C) (lanes 1–3). Whole cell lysates were prepared from cells overproducing the hemagglutinin-Cdc7 fusion protein (HA-Cdc7). Extracts were made from cells (1 l) harvested at OD_{595} = 2 by extensive manual grinding under liquid nitrogen as described by Schultz *et al.* (1991). The powder of broken cells were resuspended in L buffer and centrifuged at 20 000 rpm for 30 min. The supernatant was frozen in aliquots at −70°C until needed. The monoclonal antibody 12-CA5 (20 μl each) was used to immunoprecipitate HA-Cdc7 fusion protein from these extracts (400 μg each; lanes 4–7). Immune complexes were washed as above and incubated with (+) or without (−) 3 U of AP before assay for H1 kinase activity (lanes 4 and 5). In lane 6, immune complexes of AP-treated (1/2) and untreated (1/2) samples were combined and tested for kinase activity. Lane 7 contained immune complexes treated in the same way as those in lane 5 except okadaic acid (5 μM) was added in the kinase reaction. Reaction products were analyzed on an SDS-polyacrylamide gel, stained with Coomassie blue, dried, and autoradiographed. (B) Three separate Cdc7 immune complexes were prepared from Cdc7-overproducing cells with affinity purified Cdc7 antibody. After washing 4 times in IP buffer, 2 samples were washed twice in CIP buffer and incubated for 15 min at 37°C in the presence (+) or absence (−) of 3U of CIP (lanes 1 and 2). The third immune complex was treated with 3U of AP after washing twice in AP buffer (lane 3). Each immune complex was then subjected to immunoblot analysis as described in the legend to Figure 1.

Two-Dimensional Peptide Map Analysis of Phosphate-Labeled Cdc7 Kinase

To investigate how many phosphorylation events are involved in the regulation of Cdc7 kinase, we first addressed the question of how many phosphopeptides are found in Cdc7 protein isolated from an asynchronously growing population. Cdc7 protein was labeled with ^{32}P as described in MATERIALS AND METHODS, and purified by immunoprecipitation with affinity purified Cdc7 antibody. The Cdc7 protein was digested with trypsin and phosphopeptides were analyzed by two-dimensional electrophoresis and ascending chromatography. The protein shows four major labeled peptides (Figure 5A). This suggests that there are multiple phosphorylation sites on the Cdc7 protein and that the regulation may, therefore, be complex.

Autophosphorylation Contributes to the Phosphorylation Pattern of Cdc7 Protein

At least one of the phosphoamino acids in Cdc7 appears to be due to autophosphorylation. We have previously shown that Cdc7 protein itself becomes labeled when Cdc7 immunoprecipitates are incubated with [γ-^{32}P]ATP, suggesting that Cdc7 kinase might autophosphorylate (Yoon and Campbell, 1991). To ask if the phosphorylation is due to Cdc7 kinase or to a coimmunoprecipitating kinase, the thermolabile *cdc7-1* mutant protein was assayed for autophosphorylation. We and others have previously shown that the catalytic activity of the mutant Cdc7 kinase is thermolabile in vitro (Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991). As shown in Figure 6, the *cdc7-1* protein in yeast immunoprecipitates became phosphorylated when incubated with the ATP substrate at 25°C but not when incubated at 38°C. Because the kinase activity is thermolabile, it is most likely due to Cdc7 kinase itself and not to a contaminating kinase, which one would expect to have higher activity at 38°C. We have not yet determined the phosphopeptide map of this site, because

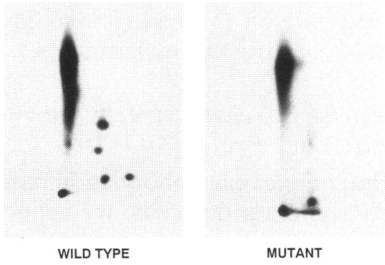

Figure 5. Tryptic phosphopeptide map of Cdc7 protein and Cdc7-Ala 83. Wild-type cells (20 ml), carrying either pSYC758 (wild-type Cdc7) or pSYC 7-83 (mutant Cdc7) were labeled as described in the legend to Figure 3B for 2 h when the A_{600} reached 0.5. After labeling, the cells pellets were resuspended in 1 ml of ice-cold buffer (50 mM Tris, pH 7.4, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 μM okadaic acid, 1 μM PMSF). One volume of glass beads was added and the cells lysed by vortexing at 4°C for 2 min. After clarifying the lysates by centrifugation, the lysates were treated with 50 μg/ml each of DNase and RNase A. Immunoprecipitation of the Cdc7 protein was carried out with 150 μl of Cdc7 antibody and 20 μl of protein A Sepharose beads (100 mg/ml). The beads were then washed 3 times with IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20, 0.5 mM EDTA) and boiled for 5 min in SDS gel loading buffer. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Tryptic phosphopeptides of Cdc7 were prepared and resolved by electrophoresis at pH 1.9 followed by ascending chromatography with phosphochromatography buffer on thin layer cellulose plates as described by Boyle *et al.* (1991). Left: Wild-type Cdc7 protein. Right: Mutant Cdc7-Ala 83 protein. Oligonucleotide mediated site-directed mutagenesis of *CDC7* was carried out as described in MATERIALS AND METHODS.

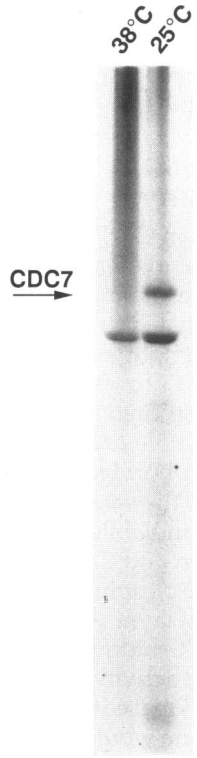

Figure 6. Cdc7 protein autophosphorylates. Plasmid pSYC7-158, containing the mutant *cdc7-1* allele, was prepared as described in MATERIALS AND METHODS. Strain cdc7-1 carrying pSYC7-158 was used to prepare nuclear extracts. Extracts were immunoprecipitated with Cdc7 antibody as described in MATERIALS AND METHODS. Protein kinase assays were carried out as described in Figure 2, except that no histone H1 was added to the reaction mixture. Reaction mixtures were incubated at either 25 or 38°C as indicated in the figure.

the amount of incorporated label is limiting and varies from experiment to experiment, perhaps due to differing levels of phosphate on the endogenous protein in extracts prepared for different experiments.

A Protein Kinase in Yeast Extracts can Phosphorylate Cdc7 Protein In Vitro

Because of the complex phosphorylation pattern of Cdc7 protein from asynchronous cells, we expect that more than one protein kinase contributes to the phosphorylation pattern. Because Cdc7 autophosphorylates, we could not easily interpret assays of yeast crude extracts for activities that phosphorylate Cdc7. Instead, we have initially focused on phosphorylation by a single kinase in yeast extracts, the Cdc28 kinase, because a number of previous findings pointed to a possible role for this enzyme. It seemed reasonable that the Cdc28 kinase or a kinase with a similar specificity might phosphorylate Cdc7, because Cdc7 carries the consensus recognition sequence for Cdc28 kinase at amino acids 83-86. Furthermore, the inactivity of Cdc7 kinase and its hypophosphorylation in *cdc28*-arrested extracts were consistent with the Cdc7 protein being a substrate of the Cdc28 kinase. The recent demonstration that Cdc28 kinase is active late in G1, that is during a *cdc7* block (Moll *et al.*, 1991), when it might be needed to activate Cdc7, was a further argument that made it reasonable to test if Cdc7 was a biochemical (or in vitro) substrate of Cdc28 kinase. To directly test this, the Cdc7 protein had to be purified so that it could be used as a substrate. Cdc7 protein was expressed in *E. coli* as a fusion protein with the hemagglutinin epitope (see MATERIALS AND METHODS for details). Although a number of different conditions were tested, the bacterially produced fusion protein, designated HA-Cdc7 protein, was not active as a kinase, in contrast to that produced in yeast (see Figure 8). In addition, Cdc7 protein expressed in *E. coli* is not phosphorylated (Yoon, Loo, and Campbell, our unpublished data). The unphosphorylated Cdc7 protein was therefore ideally useful as a substrate to test the effects of phosphorylation by Cdc28 immune complexes.

Antibodies raised against the N-terminal 11 amino acids of Cdc28 protein (gift of M. Tyers and A.B. Futcher, Cold Spring Harbor, NY) were used to prepare Cdc28 immune complexes. As shown in Figure 7, the Cdc7 fusion protein was phosphorylated by the Cdc28 immune complexes. Two controls were performed to ask if Cdc28 was responsible for the phosphorylation. First, extracts from a wild-type strain and from strain *cdc28-13*, extracts of which contain a thermolabile Cdc28 kinase (Reed *et al.*, 1985; Wittenberg and Reed, 1988), were compared as a source of Cdc28 kinase. The protein kinase reaction was carried out at the permissive (25°C) and nonpermissive temperature (38°C) for the *cdc28-*

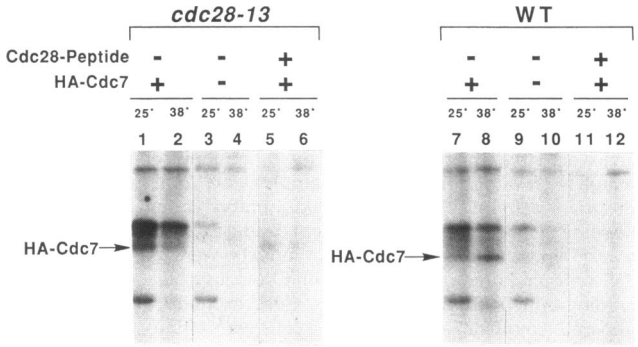

Figure 7. Phosphorylation of Cdc7 protein by Cdc28 immune complexes. Whole yeast lysates were prepared from a *cdc28-13* strain and a wild-type strain as described in the legend to Figure 4. Cdc28 kinase was then immunoprecipitated from these extracts (100 μg each) with excess antibody (2 μl each) raised against the N-terminal 11 amino acids of Cdc28 protein (lanes 1–4 and 7–10) or with the same antibody preincubated with 30 μg of the N-terminal peptide (lanes 5, 6, 11, and 12). Cdc28 immune complexes were prepared from *cdc28-13* strain in lanes 1–6 and from wild-type strain in lanes 7–12. Kinase reactions were carried out at 25°C (lanes 1, 3, 5, 7, 9, and 11) or 38°C (lanes 2, 4, 6, 8, 10, and 12) with 500 ng of HA-Cdc7 fusion protein as substrate. The fusion protein was purified out of *E. coli* cells as explained in MATERIALS AND METHODS. In lanes 3, 4, 9, and 10, Cdc28 immune complexes were incubated in the absence of the fusion protein. Products were analyzed by electrophoresis on a 10% SDS-polyacrylamide gels, which were run until the 30-kDa marker protein reached the bottom. The HA-CDC7 band was identified by staining of the gels with Coomassie blue. The 500 ng of HA-Cdc7 fusion protein was the major band and was absent where HA-Cdc7 was omitted from the experiment. Lanes 1–6 were run on one gel and lanes 7–12 on another gel. The experiments with the *cdc28-13* extract were repeated 4 times.

13 mutation. Comparison of lanes 1 and 2 in Figure 7 demonstrates that there was less Cdc7 phosphorylating activity in the immune complexes prepared from strain *cdc28-13* at 38°C than at 25°C. In contrast, immune complexes from the wild-type strain, while less active overall, were more active at 38°C than at 25°C (lanes 7 and 8). As a second control, the 11 amino acid, Cdc28-derived peptide employed as immunogen was used as competitor in the immunoprecipitation. Cdc7 was not phosphorylated when the Cdc28 antibody was preincubated with the Cdc28-peptide (Figure 7, lanes 5, 6, 11, and 12). Thus the in vitro phosphorylation is likely due to the Cdc28 protein.

Additional proteins were phosphorylated by the Cdc28 immune complexes as shown in Figure 7. None of these proteins appeared to be phosphorylated in a Cdc28-specific way, because the level of phosphorylation was the same at either temperature or the kinase activity was thermolabile even in the wild-type strain. However, the ~62 kDa band present above the HA-Cdc7 protein could represent a coprecipitating cyclin.

Similar results to those shown in Figure 7 have been obtained by using p13 beads (gift of W.G. Dunphy; Dunphy and Newport, 1989) and Cks1 antibodies (gift of C. Wittenberg) (unpublished observation) to prepare the Cdc28 kinase. *CKS1* encodes the *S. cerevisiae* p13 analog and Cks1 antibodies have been shown to coimmunoprecipitate active Cdc28 kinase along with Cks1 protein (Hadwiger *et al.*, 1989a). Because the enzymological specificity of Cdc28 present in Cdc28 kinases prepared with these different reagents has not been characterized, we cannot yet derive any information about which of the postulated forms of Cdc28 kinase is active in our experiments.

The phosphorylation by Cdc28 in vitro is not efficient enough currently to allow mapping of the peptide(s) directly phosphorylated by the Cdc28-like activity. This appears to be due to difficulty and lack of reproducibility in refolding the protein purified from *E. coli* (see MATERIALS AND METHODS). We have been unable to increase the level of phosphorylation of HA-Cdc7 fusion protein as compared with that of other proteins, due to difficulties thus far in obtaining concentrated HA-Cdc7 protein in soluble form. The fusion protein precipitates at concentrations >50 ng/μl under reaction conditions tested thus far. Although we have not been able to achieve the sensitivity necessary to carry out phosphopeptide analysis of the Cdc7 protein phosphorylated in vitro, an alternative approach to gaining information about the amino acid site phosphorylated by the Cdc28 immunoprecipitates is provided by the ability to express a mutant Cdc7 protein with an altered Cdc28 consensus recognition site in yeast. A two-dimensional phosphotryptic peptide map of Cdc7 protein containing a mutation of Ser 83 to Ala, in the putative Cdc28 consensus recognition site, is shown in Figure 5B. Comparison to the wild-type protein in Figure 5A shows that there is a major alteration in the phosphorylation pattern; two spots are greatly reduced in intensity and a third spot is missing. Protein blotting verifies that the amount of mutant protein in extracts is identical to that of wild type (Loo and Campbell, unpublished observations). Thus the in vivo pattern suggests that alteration of the consensus site for Cdc28 phosphorylation has a dramatic effect on more than one phosphorylation site on the Cdc7 protein. We cannot make a definitive statement about how many phosphorylation sites are affected until we know whether the three spots in wild type arise due to multiple, distinct phosphorylation sites or due to incomplete typtic digestion. Further studies will be required to show if this biochemical result reflects the physiological situation.

Phosphorylation of Cdc7 Protein by Cdc28 Activates the Cdc7 Kinase

To test whether phosphorylation of Cdc7 by Cdc28 is sufficient to activate the inactive bacterially produced Cdc7 protein, we designed an experiment in which we first phosphorylated highly purified, soluble, bacterially produced HA-Cdc7 protein with cold ATP with Cdc28 immune complexes from crude yeast extracts. We then removed the Cdc28 kinase and assayed the ability of the Cdc7 kinase to phosphorylate histone H1. To insure that Cdc28 could not participate in the histone H1 phosphorylation reaction, we used extracts prepared from a *cdc28-13* strain that is known to produce a kinase that is thermolabile in vitro. The first reaction was carried out at 23°C, where the *cdc28-13* kinase is active, and the second was carried out at 37°C, where the *cdc28-13* kinase is not active. If wild-type *CDC28* extracts were used, sufficient Cdc28 kinase activity remained in the supernatant so that even in the control without HA-CDC7 protein added, there was some histone H1 kinase activity. As shown in Figure 8, HA-Cdc7 kinase produced in *E. coli* is active only after treatment with Cdc28 kinase (compare lanes 2 and 3). Inclusion of the Cdc28 peptide during preparation of Cdc28 immune complexes abolished the activation (lane 3) and activation was severely reduced at high temperature when *cdc28-13* was used as source of the Cdc28 protein (lane 4). Thus we conclude that Cdc7 phosphorylation by Cdc28 can activate the kinase activity, though potential contribution of other components in the Cdc28 immunoprecipitates does not allow the conclusion that Cdc28 is sufficient to activate Cdc7. [A report, that might appear to conflict with our findings, that Cdc7 was active as a kinase when prepared in an in vitro translation system, in which posttranslational modification is not expected to occur, could be explained by the adventitious presence of a Cdc28-like kinase in the translation kit (Bahman *et al.*, 1988).]

DISCUSSION

Our studies were initiated in an attempt to define the molecular basis of the timing of Cdc7 function in the

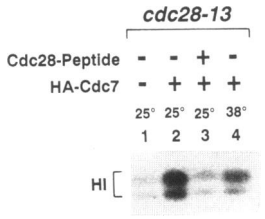

Figure 8. Cdc28 kinase activates Cdc7 kinase in vitro. This experiment represents a 2-stage in vitro phosphorylation protocol. In stage 1, Cdc7 is phosphorylated by Cdc28 immune complexes. In stage 2, histone H1 is phosphorylated by the Cdc7 kinase. Stage 1: Cdc28 immune complexes were prepared from strain *cdc28-13* as described in the legend to Figure 5. In lane 3, the Cdc28 antibody was preincubated with 30 μg of the Cdc28 peptide as competitor. After 5 min at 25°C (lanes 1–3) or 38°C (lane 4), either 20 μl of HA-Cdc7 fusion protein (50 ng/ml) in buffer B (lanes 2–4) or 20 μl of buffer B alone (lane 1) were added together with 1 mM ATP. Incubation was continued at the same temperature for 10 min and reaction mixtures were centrifuged for 1 min to remove Cdc28 immunoprecipitates. Stage 2: supernatants, which contained either HA-Cdc7 protein or buffer alone, were incubated at 38°C for 5 min to inactivate any residual *cdc28-13*-dependent kinase. Then, histone H1 kinase reactions were carried out at 38°C for 10 min with 0.1 mM ATP, 10 μCi [γ-^{32}P] ATP, and 5 μg of histone H1. Reaction products were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

yeast cell cycle. Demonstration that *CDC7* encodes a protein kinase (Patterson *et al.*, 1986; Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991) provided us with an assay for factors that contribute to the regulation of the Cdc7. Biochemical experiments showed that Cdc7 kinase activity fluctuates in the cell cycle with a periodicity coinciding with the in vivo function of Cdc7, but the steady-state protein levels remain constant, suggesting that it is the kinase activity of Cdc7 that is responsible for its essential role and that it is activation rather than abundance that accounts for cell cycle variation. In addition, we showed that Cdc7 kinase activity is undetectable in extracts of cells arrested before START, either by an α-factor block or by a *cdc28* mutation, and found that this corresponds to a period when the Cdc7 protein is hypophosphorylated compared with any other point in the cell cycle. Combined with the fact that dephosphorylation of Cdc7 in vitro reduces the kinase activity to pre-START levels, these findings suggest that phosphorylation plays a role in regulating Cdc7 function. In an effort to specify the important phosphorylation events, we then showed that there are at least four tryptic phosphopeptides in Cdc7 protein populations. Both autophosphorylation and phosphorylation in trans by a protein in yeast extracts occur. The ubiquitous protein kinase, Cdc28 kinase, is at least one protein that can phosphorylate Cdc7 protein in vitro. These observations raise many interesting questions about the timing of multiple phosphorylations of Cdc7, the role of individual phosphorylations and their interdependence and whether Cdc7 is a substrate of the Cdc28 kinase in vivo.

Timing of Cdc7 Function in the Cell Cycle

Early work from the Hartwell laboratory defined three genes necessary for the initiation of DNA replication, *CDC28, CDC4,* and *CDC7,* as discussed in the INTRODUCTION. *CDC28* functions at START, and after return of *cdc28* mutants from the nonpermissive temperature the cells can still adopt the alternative pathway of conjugation. Several more genes that have this phenotype have been identified but as yet have not been well studied. Three additional genes that affect START, *CLN1, 2,* and *3,* are apparently involved in a positive feedback loop controlling the activation of the *CDC28* gene product (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). These genes were identified either as suppressors of *cdc28* mutations (*CLN1, CLN2*) or as dominant gain of function mutations (*CLN3*) (Sudbery *et al.*, 1980; Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989b) and have at least partially redundant functions, in that one *CLN* gene can compensate for loss of the other, but loss of all three is lethal (Richardson *et al.*, 1989). The *CLN* genes encode proteins that are distantly related to the cyclins that activate the p34/cdc2$^+$/MPF kinase for induction of mitosis in *Xenopus, S. pombe* and other organisms. By analogy, it has been proposed that *CLN1, 2,* and *3* interact with and activate the Cdc28 kinase subunit and passage through START (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; see also Cross, 1988; Nash *et al.*, 1988 and for review Nasmyth, 1990). The molecular nature of the events set in motion by these START genes, however, has been more difficult to define.

Morphological analysis of single and double mutants and reciprocal shift studies suggest *CDC7* acts subsequent to *CDC28*/START (Hereford and Hartwell, 1974). In addition to *CDC4* and *CDC7,* three other genes, *CDC34, DBF4,* and possibly *DBF2* also function in the post-START/pre-DNA synthesis period (Johnston and Thomas, 1982; Goebl *et al.*, 1988; Chapman and Johnston, 1989; Johnston *et al.*, 1990). These dependent genetic relationships predicted that activation of the Cdc7 protein should be dependent on completion of START. In keeping with this, we found that Cdc7 kinase was inactive in extracts of cells arrested at START—either by α-factor or by a *cdc28* arrest. We then found that after release from a block and passage through START the kinase activity increased, decreased abruptly late in the cell cycle, and then rose again in G1 in the second cell cycle. This temporal correlation of appearance of kinase activity and in vivo function adds to the argument, previously based solely on site-directed mutagenesis (Buck *et al.*, 1991; Hollingsworth *et al.*, 1992), that the kinase activity of Cdc7 is related to its essential and periodic function.

Genetic studies predicted that Cdc7 activity might be dependent on *CDC4,* also. The results in Figure 2 suggest that, on the contrary, the Cdc7 kinase is already active

even before *CDC4* completes its function. Why does *CDC7* appear to depend upon the completion of *CDC4* in vivo? One possibility is that the substrates of Cdc7 kinase may not be available during the *cdc4* block. According to the results of Hereford and Hartwell (1974), *cdc4* mutants blocked and released in the presence of the protein synthesis inhibitor cycloheximide do not enter S phase. Further protein synthesis is thus necessary after the *CDC4* step. Proteins newly synthesized after the *CDC4* step may include replication proteins that require the Cdc7 kinase for posttranslational activation. Alternatively, substrates of Cdc7 kinase may exist during the *cdc4* block, but somehow may be inaccessible to Cdc7 protein until after *CDC4* has functioned. A third possibility is that Cdc7 kinase, like many other kinases, actually consists of two subunits, a catalytic subunit and a regulatory subunit whose abundance or modification is dependent on *CDC4* function. We feel that it is likely that Cdc7 does have a regulatory subunit that enhances activity and is encoded by a second yeast gene, since we have recently been able to purify an active Cdc7 kinase from yeast and have found that Cdc7 copurifies with an 80-kDa protein, though we have not yet determined whether this 80-kDa protein represents another substrate or an essential cofactor for Cdc7 activity (Loo and Campbell, unpublished observations). We also observe an 80-kDa protein in Cdc7 immunoprecipitates from yeast (see Figure 3, Yoon and Campbell, 1991). Genetic suppression of *cdc7* by overproduction of *DBF4* and vice versa could also be interpreted as indicating that the Cdc7 kinase has a second subunit (Kitada *et al.*, 1992).

How is the timing of Cdc7 action controlled? One component of the regulation seems to be phosphorylation of Cdc7 protein. Phosphatase treatment of Cdc7 kinase abolishes its histone H1 kinase activity (Figure 4). In addition, its phosphorylation state correlates well in the cell cycle with the time when it is active (Figures 2 and 3). It appears likely from our studies that Cdc28 kinase provides at least one essential phosphate modification (Figures 5 and 6). We also know that Cdc7 is phosphorylated by at least one other kinase, in that Cdc7 appears to autophosphorylate (Figure 3 in Yoon and Campbell, 1991; and this work Figure 6).

Possible Roles for Phosphorylation in Cdc7 Activation

Dephosphorylation of Cdc7 immune precipitates leads to their inactivation as a kinase. This suggests that at least one phosphoamino acid is necessary for catalytic activity. We cannot rule out the more trivial explanation that dephosphorylation causes dissociation of an as yet unidentified, essential subunit. Even if the latter is the case, however, ultimately the phosphorylation event is essential for activation and therefore crucial. Thus we have begun an analysis of the phosphorylation of Cdc7 protein. We have now provided strong evidence that Cdc7 autophosphorylates. In their studies of cell cycle phosphorylation of $p34^{cdc2}$ kinase, Krek and Nigg (1991) found that it is probably Thr 161 that is phosphorylated in M phase and pointed out that Thr 161 is located within a conserved domain which in other kinases is subject to autophosphorylation (Hanks *et al.*, 1988). Furthermore, they referred to the fact that the cognate amino acid, Thr 167, in *S. pombe* cdc2 kinase is essential for function and is phosphorylated. The corresponding residue in Cdc7 kinase is Thr 281, located in region VIII of the canonical protein kinase active site (Hanks *et al.*, 1988). Buck *et al.* (1991) have shown by site-directed mutagenesis that either a serine or a threonine at this position is essential for the in vivo functions of *CDC7*. Therefore, we predict that Thr 281 may be the site of autophosphorylation and that phosphorylation of this site may be required for full Cdc7 activity. We note, however, that *Xenopus* $p34^{cdc2}$, which requires phosphorylation of Thr 161 for activation, does not appear to autophosphorylate but rather to be phosphorylated in trans (Solomon *et al.*, 1992).

Is CDC7 a Physiological Substrate of CDC28?

We have shown that Cdc28 immunoprecipitates can phosphorylate and even activate the histone H1 phosphorylating activity of Cdc7 kinase in vitro and that Cdc7 kinase is inactive in cells blocked by a *cdc28* mutation. In keeping with our proposal that autophosphorylation is necessary for activity, then phosphorylation by the "Cdc28" kinase may change the conformation of Cdc7 to a state that allows autophosphorylation of an additional essential site. It has been shown for several kinases that phosphorylation of one site can influence sequential phosphorylation of other sites (see Roach, 1991, for complete discussion of multisite and hierarchical protein phosphorylation). Indeed, our phosphopeptide analysis suggests that there may be at least four phosphoamino acids on the Cdc7 protein. Krek and Nigg (1991) have discovered four phosphorylation sites on mammalian $p34^{cdc2}$ and have shown that each one is cell cycle regulated: Thr 14 and Tyr 15 are phosphorylated in G2; Thr 161, probably, is phosphorylated in M, and Ser 277 phosphorylation peaks in G1. All probably contribute to the regulation of the enzyme. Considering the multiple functions of Cdc7, similar complex regulation is not unlikely.

Although we also favor a multisite phosphorylation scenario for Cdc7, we by no means claim that our results establish Cdc7 as a substrate of Cdc28 in vivo. Given the nature of cell cycle mutants, one could argue that the effect of the *cdc28* mutation is indirect. Another intermediate kinase may be required or an essential cofactor may not become available until after START. For instance, Solomon *et al.* (1992) have shown that *Xenopus* $p34^{cdc2}$ must associate with cyclin to be phosphorylated

on Thr 161. Therefore the reduction in phosphorylation in the *cdc28* mutant does not constitute an argument that Cdc28 is a Cdc7 kinase in vivo. Furthermore, our demonstration that phosphorylation of Cdc7 in vitro by Cdc28 activates Cdc7 kinase is important, but it is inconclusive in the sense that it only demonstrates that the kinase active in vivo must have the same specificity as Cdc28. The consensus motif for a $cdc2^+$/Cdc28 phosphorylation site has been reported to be S/T-P-X-Z (where X is a polar amino acid, and Z is generally a basic amino acid) (for reviews, see Lewin, 1990; Moreno and Nurse, 1990 and Pines and Hunter, 1990). The primary amino acid sequence of *CDC7* contains one such motif, $TSS^{83}PQR$, near the N-terminus, adjacent to the ATP binding site. Mutation of Ser 83 to Ala 83 results in a drastically altered phosphopeptide map of Cdc7 (Figure 5, this work). Furthermore, we have recently expressed the Ala 83 Cdc7 mutant protein in the *cdc7-1* strain and found that it failed to complement the *cdc7-1* mutant for growth at the nonpermissive temperature (Loo, Yoon and Campbell, unpublished observations). Taken together these results are consistent with Ser 83 being the site of a crucial phosphorylation. In some cases, even the simple sequence S/T-P is recognized by $cdc2^+$ kinases (see references in Roach, 1991). Cdc7 contains three SP or TP sites which lie in the C terminal half of Cdc7. Mutations of the respective serines and threonines in these positions have no effect on in vivo function (Loo, Yoon, and Campbell, unpublished observations).

Recently, three criteria have been set for establishing a protein as the physiological substrate of a particular kinase (Lewin, 1990). First, the amino acid phosphorylated by the kinase in vitro should be occupied in vivo at the point in the cell cycle when it is needed. Second, mutations, either in the kinase or in its recognition site in the substrate or both, should block phosphorylation in vivo. Third, some function of the protein should be altered by phosphorylation. To identify the kinases that phosphorylate Cdc7 and their sequence of action will require much more work: mapping of specific phosphorylation sites, determination of function of specific phosphorylation events by site directed mutagenesis, evaluation of phosphopeptide maps in protein kinase mutants and purification of the kinases responsible. Only when all this work is done will we be able to answer whether and or how sequential phosphorylation influences Cdc7's various in vivo functions.

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